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# HYDROCARBON BIODEGRADATION: BIOCHEMICAL CHARACTERIZATION OF BACTERIA ISOLATED FROM LOCAL SOILS

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Fifty-six bacterial isolates were separated and purified from fourteen soil samples collected from ecologically different environments in Lahore. Thirty-seven of these were gram-negative and nineteen gram-positive. The growth pattern of the gram-negative organisms were examined using different hydrocarbons as energy source. Six of these isolates efficiently utilized chicken fat as the sole source of energy. Those isolates which exhibited oxidase-positive response were characterized as *Pseudomonas aeroginosa* and P. strutzeti. The remaining oxidase-negative isolates were characterized as Enterobacter sakazaki and E. cloacae. When chicken fat was replaced with kerosene oil as the carbon source, the Ps22 and PS 37 isolates produced emulsification.

*Key words:* Bacterial isolates, Lacal soils, Biodegradations.

## INTRODUCTION

A variety of microorganisms have the ability to utilize simple hydrocarbons as the sole sources of energy and carbon [10]. The utilization of hydrocarbon is highly dependent on its chemical nature [10]. Nevertheless, these microorganisms can be exploited for their ability to eliminate oil pollution and also in the tertiary recovery of crude oil from petroleum oil wells [5,9,2].

Bacteria belonging to the genus Pseudomonas produce, as part of their normal metabolic functions, powerful emulsifiers or surfactants [2] which have commercial applications in the dewaxing and oil cleaning industry [2]. Production of such compounds often depends on the hydrocarbon used as substrate. However, chicken fat is a preferred substrate for studies on the production of surfactant and emulsifier [2].

The present studies form a part of the culture collection programme of this Centre, to screen local environments for efficient degraders of long chain hydrocarbons. A large number of microorganisms have been isolated from soil samples collected from ecologically most suitable sites in Lahore city. Isolated bacteria have been taxonomically identified and biochemically characterized. It is reported that isolated strains utilize hydrocarbon and produce surfactants and emulsifiyers.

## MATERIAL AND METHODS

*Collection of soil samples:* Soil samples continuously contaminated with oil and petrol were collected from fourteen different petrol pumps in Lahore. The soil around the opening valve of the petrol reservoir was scooped into sterile screw cap tubes.

*Isolation of bacteria.* Soil samples were mixed with an equal quantity of sterile physiological saline and the slurry was inoculated on the nutrient agar (Difco) plates, enriched with 5 % yeast extract. After incubation at $32^{o}$ for 48 hr bacterial colonies appearing were separated and restreaked to single colony isolates. Every bacterial colony was examined for its size, shape, texture, growth rate and gram-staining reaction.

*Drug resistance:* Resistance to ampicillin (30 $\mu$g/ml) was examined by growing the cells on L.B. Agar medium containing the required concentration of the drug.

*Study of growth on hydrocarbon substrates:* Different isolates were grown in buffered mineral medium as suggested by Mills *et. al.* [8] containing one of the six different hydrocarbon substrates, chicken fat, paraffin oil, liquid paraffin, crude oil, furnace oil and Mobil oil. Bacterial growth was graded according to the level of O.D. at 540nm. Cultures with O.D. above 0.600 and below 0.300 were graded as A and C respectively. Isolates showing good growth on all of the six hydrocarbon substrates were further studied.

*Characterization:* Bacterial isolates falling under category 'A' above were characterized according to the method of Kreig and Holt [7]. Entero-tubes and oxi-ferm tubes (Roche) were used for fermentation test of adonitol, lactose, arabinose, sorbitol, dulcitol, maltose, mannitol, sucrose and xylose; decarboxylation of lysine, ornithine,

arginine; formation of $H_2S$ and $N_2$; deamination of phenylalanine; hydrolysis of urea and utilization of sodium citrate.

Oxidase reagent (tetramethyl-*p*-phenylene diamine) was used to detect oxidase reaction of the isolates and gelatin liquification was observed in the nutrient gelatin slants.

*Emulsification:* Emulsification was studied by shaking 5 ml of a 10 day old culture with equal quantity of kerosene oil [3] and allowing it to stand for 48 hrs. The appearance of an emulsified interface was measured.

## RESULTS AND DISCUSSION

Fifty-six isolates were separated from fourteen soil samples collected from different locations in Lahore. Gram-staining studies show that thirty-seven of these isolates were gram-negative and the remaining nineteen were gram-positive. Since the ability to degrade petroleum hydrocarbon is generally confined to gram-negative bacteria therefore, only gram-negative isolates were further studied [1, 6].

Resistance to ampicillin, being one important selection marker, was examined (Table 1). Results in Table 1 present resistance to 30$\mu$g/ml of ampicillin in L.B. medium. The data show that thirteen isolates were resistant whereas the remaining fifteen were sensitive to the drug. These findings are expected to help in the genetic manipulation of hydrocarbon degrading genes when ampicillin resistance can be used as a selection marker.

Growth behaviour of the isolates was measured in buffered mineral medium [8] in the presence of one of the six different hydrocarbon substrates, namely chicken fat. Table 1 shows that twenty-eight out of the total thirty seven isolates showed growth on chicken fat and other hydrocarbons.

According to Atlas [1] the exposure of isolates to refined fractions of petroleum yields non-predominant isolates capable of utilizing crude oil, furnace oil, and mobil oil. The results of present study seen in good agreement. When mobil oil, furnace oil and crude oil were used as substrates some isolates gave B and C levels of growth whilst others did not grow at all. In the case of liquid paraffin and paraffin oil only Ps3, Ps12, Ps18, Ps26 and Ps37 gave A level growth. In the case of chicken fat almost all isolates except for Ps3, Ps5, Ps23, Ps26, Ps41 and Ps46 give good growth.

Ps2, Ps11, Ps12, Ps22, Ps37 and Ps40 showed best growth on chicken fat. Growth patterns of these isolates are shown in Fig. 1. The individual growth patterns of six isolates follow the usual bacterial growth curve except for minor changes which may be accounted for experimental errors with Ps12 and Ps40, which being fast growers gave the maximum growth on the 3rd day whilst others grew to a maximum density on the 4th or 5th day. This is followed by a stationary phase which continues uptil the 7th day of almost all the isolates after which a decline phase continues.

These six isolates were further identified according to the method of Kreig and Holt [7] in *Manual of Systematic Bacteriology*. Results are presented in Table 2 and 3.

Three oxidase-psotive isolates are taxonomically characterized as *Pseudomonas aerogenosa* (Ps2), *P. strutzeti* (Ps11 and Ps37). The remaining oxidase negative isolates belong to Enterobacter family. Ps22 and Ps12 are characterized as *Enterobacter sakazaki* and Ps40 as *E. cloacae.*

Chakarbarty [2] discovered a strain of *Pseudomonas*

Table 1. Ampicillin resistance and biodegradation of different hydrocarbons by 28 gram-negative strains isolated from the soil of petrol pumps.

| Strain | Ps 2 | Ps 3 | Ps 5 | Ps 11 | Ps 12 | Ps 14 | Ps 18 | Ps 19 | Ps 22 | Ps 23 | Ps 26 | Ps 27 | Ps 28 | Ps 30 | Ps 32 | Ps 34 | Ps 35 | Ps 36 | Ps 37 | Ps 38 | Ps 40 | Ps 41 | Ps 42 | Ps 43 | Ps 44 | Ps 45 | Ps 47 | Ps 55 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ampicillin resistance | $A^S$ | $A^r$ | $A^r$ | $A^r$ | $A^S$ | $A^r$ | $A^r$ | $A^r$ | $A^S$ | $A^S$ | $A^S$ | $A^r$ | $A^S$ | $A^r$ | $A^r$ | $A^r$ | $A^S$ | $A^S$ | $A^S$ | $A^S$ | $A^S$ | $A^r$ | $A^S$ | $A^S$ | $A^S$ | $A^S$ | $A^r$ | $A^r$ |
| Chicken fat | A | C | B | A | A | A | A | A | A | B | C | A | A | C | A | A | A | A | A | A | A | B | A | A | A | A | C | A |
| Liquid paraffin | – | C | – | – | A | – | – | – | – | – | – | – | – | – | C | C | B | C | – | – | – | – | C | C | – | – | – | B |
| Paraffin oil | – | A | – | – | B | C | A | – | B | B | A | – | C | C | C | – | B | C | A | B | C | – | – | – | – | – | – | – |
| Mobil oil | C | – | – | – | C | – | A | – | – | – | – | C | – | C | – | C | – | A | A | C | A | – | B | B | – | A | – | – |
| Furnace oil | C | – | – | C | – | – | C | B | – | – | – | – | – | – | – | – | – | B | – | – | – | – | B | B | A | C | – | B |
| Crude oil | – | – | C | – | – | C | – | – | – | – | – | – | B | – | – | – | – | – | C | – | C | – | – | – | – | – | – | B |

$A^r$: ampicillin resistant. $A^S$: ampicillin sensitive.

Hydrocarbon degradation is noted in terms of optical density at 540 nm. A: 0.600 units or above, B: 0.300-0.600 units and B: below 0.300.

Table 2. Properties of oxidase-positive strains

| Properties | Strains Ps11 | Ps2 | Ps37 |
|---|---|---|---|
| Fermentation of: | | | |
| Glucose (anaerobic) | – | – | – |
| Glucose(aerobic) | + | + | + |
| Lactose | – | – | – |
| Xylose | + | + | + |
| Maltose | – | – | – |
| Mannitol | – | – | – |
| Sucrose | – | – | – |
| Decarboxylation of: | | | |
| Arginine | – | + | – |
| Lysine | – | – | – |
| Formation of nitrogen | + | – | + |
| Production of indole | – | – | – |
| Hydrolysis of urea | – | + | – |
| Deamination of phenylalanine | – | – | – |
| Utilization of sodium citrate | + | + | + |
| Pigmentation | No Pigmentation | Green | No Pigmentation. |
| Gelatin liquefaction. | – | + | – |

Table 3. Properties of oxidase-negative trains.

| Properties | Strains Ps40 | Ps12 | Ps22 |
|---|---|---|---|
| Fermentation of: | | | |
| Glucose | + | + | + |
| Adonitol | – | – | – |
| Lactose | + | + | + |
| Arabinose | + | + | + |
| Sorbitol | – | – | – |
| Dulcitol | + | – | – |
| Formation of: | | | |
| Gas | – | + | + |
| $H_2S$ | – | – | – |
| Decarboxylation of: | | | |
| Lysine | – | – | – |
| Ornithine | + | + | + |
| Deamination of Phenylalanine | – | – | – |
| Hydrolysis of urea | – | – | – |
| Utilization of Na-citrate | + | + | + |
| Pigmentation | No Pigmentation | Yellow colonies | Yellow colonies |
| Gelatin liquefaction | – | + | + |

*aeroginosa* which produces 400 units/ml of emulsifying agent. It has been observed that Ps22 and Ps37 produce an emulsification layer when chicken fat was replaced with

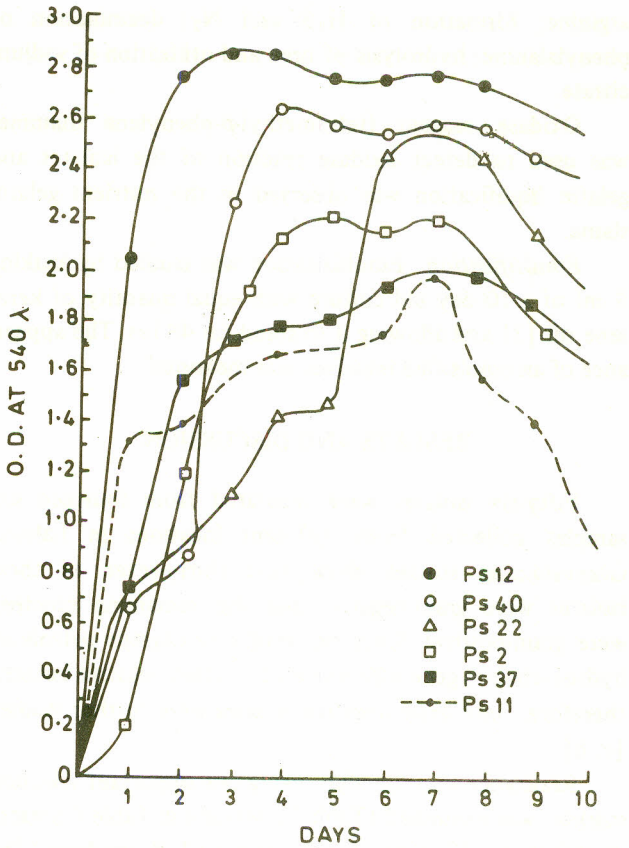


Fig. 1. Growth pattern of Ps2, Ps11, Ps12, Ps22, Ps37 and Ps40 on chicken fat.

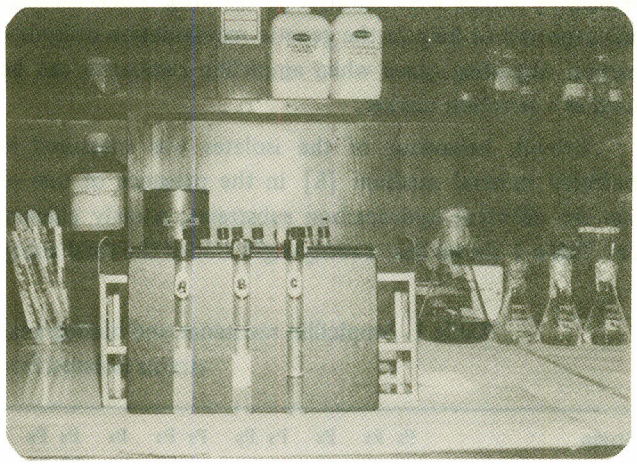

Fig. 2. Study of emulsifying agents.
(A). Ps22, (B) Ps37, (C) Control (tween 80).

kerosene oil as a source of carbon and energy. Different emulsification layers are produced by Ps22 and Ps37 with a one-inch long layer of loose bubbles in the case of Ps22 and a denser but shorter layer for Ps37 in comparison to no layer when tween 80 is used as a control (Fig. 2). Thus it is suggested that strains of *Enterobacter sukazaki and Pseudomonas strutzeti* produce an emulsification layer on kerosene oil.

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